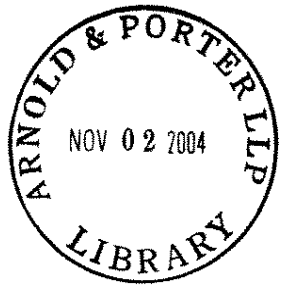
APPENDIX 5

2005

USP 28

NF 23



THE UNITED STATES PHARMACOPEIA

THE NATIONAL FORMULARY

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UNITED STATES PHARMACOPEIAL CONVENTION, INC.
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Test solution—After 120 minutes, withdraw a portion of the solution under test, filter, and dilute with *Medium*, if necessary, to obtain a solution having an estimated concentration of about 0.28 mg of erythromycin per mL.

Procedure—Transfer 5.0-mL portions of the *Working standard solution* to two 25-mL volumetric flasks, one of which serves as a working standard blank. Similarly, transfer 5.0-mL portions of the *Test solution* to two 25-mL volumetric flasks, one of which serves as a blank for that *Test solution*. To each of the flasks designated as a blank add 2.0 mL of 0.5 N sulfuric acid and to the remaining flasks add 2.0 mL of water. Allow to stand for 5 minutes with intermittent swirling. To all flasks add 15.0 mL of 0.25 N sodium hydroxide, dilute with *Medium* to volume, and mix. Heat the flasks in a water bath at 60 ± 0.5° for 5 minutes, and allow to cool. Using a suitable spectrophotometer, determine the absorbance of each solution, corrected for its blank solution, at the wavelength of maximum absorbance at about 236 nm. Determine the amount of $C_{37}H_{67}NO_{13}$ dissolved from the *Test solution* in comparison with the solution obtained from the *Working standard solution*.

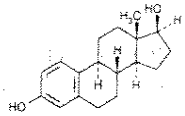
Tolerances—Not less than 75% *(Q)* of the labeled amount of $C_{37}H_{67}NO_{13}$ is dissolved in 120 minutes.

Uniformity of dosage units ⟨905⟩: meet the requirements.

Loss on drying ⟨731⟩—Dry about 100 mg of powdered Tablets in a capillary-stoppered bottle in vacuum at 60° for 3 hours: it loses not more than 5.0% of its weight.

Assay—Proceed with Tablets as directed in the *Assay* under *Erythromycin Tablets*.

Estradiol



$C_{18}H_{24}O_2$ 272.39
Estra-1,3,5(10)-triene-3,17-diol, (17β)-.
Estra-1,3,5(10)-triene-3,17β-diol [*50-28-2*].

» Estradiol contains not less than 97.0 percent and not more than 103.0 percent of $C_{18}H_{24}O_2$, calculated on the anhydrous basis.

Packaging and storage—Preserve in tight, light-resistant containers. Store at 25°, excursions permitted between 15° and 30°.

USP Reference standards ⟨11⟩—*USP Estradiol RS. USP Estrone RS.*

Identification—
A: *Infrared Absorption* ⟨197M⟩.
B: *Ultraviolet Absorption* ⟨197U⟩—
Solution: 50 µg per mL.
Medium: alcohol.
Absorptivities at 280 nm, calculated on the anhydrous basis, do not differ by more than 3.0%.

Melting range, *Class I* ⟨741⟩: between 173° and 179°. [NOTE—Dry over silica gel for not less than 16 hours prior to testing.]

Specific rotation ⟨781S⟩: between +76° and +83°.
Test solution: 10 mg per mL, in dioxane.

Water, *Method I* ⟨921⟩: not more than 3.5%.

Chromatographic purity—[NOTE—Make all solutions fresh daily.]
Mobile phase—Prepare a filtered and degassed mixture of 2,2,4-trimethylpentane, *n*-butyl chloride, and methanol (45 : 4 : 1). Make adjustments if necessary (see *System Suitability* under *Chromatography* ⟨621⟩).
Diluting solution—Prepare a filtered and degassed mixture of *n*-butyl chloride and methanol (5 : 1).
Test solution—Transfer about 70 mg of Estradiol, accurately weighed, to a 10-mL volumetric flask, dissolve in *Diluting solution*, shake vigorously to aid dissolution, dilute with *Diluting solution* to volume, and mix.
Chromatographic system (see *Chromatography* ⟨621⟩)—The liquid chromatograph is equipped with a 280-nm detector and a 4.6-mm × 25-cm column that contains packing L3. The flow rate is about 2 mL per minute. Chromatograph the *Test solution*, and record the peak responses as directed for *Procedure:* the resolution between estradiol and any impurity is not less than 1.0; the column efficiency is not less than 800 theoretical plates; the tailing factor is not more than 1.5; and the relative standard deviation for replicate injections is not more than 2.0%.
Procedure—Inject a volume (about 10 µL) of the *Test solution* into the chromatograph, record the chromatogram, and measure the peak responses. Calculate the percentage of each impurity in the portion of Estradiol taken by the formula:

$$100(r_i / r_s),$$

in which r_i is the peak response for each impurity; and r_s is the sum of the responses of all the peaks: not more than 0.5% of any individual impurity is found; and not more than 1.0% of total impurities is found.

Assay—
Mobile phase—Prepare a filtered and degassed mixture of acetonitrile and water (55 : 45). Make adjustments if necessary (see *System Suitability* under *Chromatography* ⟨621⟩).
Internal standard solution—Transfer about 300 mg of ethylbenzene to a 500-mL volumetric flask, add methanol to volume, and mix.
Standard preparation—Dissolve accurately weighed quantities of USP Estradiol RS and USP Estrone RS in methanol to obtain a solution containing 0.40 mg and 0.24 mg, respectively, in each mL. Pipet 10 mL of this solution and 5 mL of the *Internal standard solution* into a 200-mL volumetric flask. Add 100 mL of methanol, dilute with water to volume, and mix to obtain a solution having a known concentration of about 20 µg of USP Estradiol RS per mL.
Assay preparation—Transfer about 100 mg of Estradiol, accurately weighed, to a 250-mL volumetric flask, add methanol to volume, and mix. Transfer 10.0 mL of this solution to a 200-mL volumetric flask, add 5.0 mL of *Internal standard solution* and 100 mL of methanol, dilute with water to volume, and mix.
Chromatographic system (see *Chromatography* ⟨621⟩)—The liquid chromatograph is equipped with a 205-nm detector and a 3.9-mm × 30-cm column that contains packing L1. The flow rate is about 1 mL per minute. Chromatograph the *Standard preparation* and record the peak responses as directed for *Procedure:* the retention times are about 0.7 for the internal standard, about 1.3 for estrone, and 1.0 for estradiol; the resolution, *R*, between the estradiol and estrone is not less than 2.0; and the relative standard deviation for replicate injections is not more than 2.0%.
Procedure—Separately inject equal volumes (about 25 µL) of the *Standard preparation* and the *Assay preparation* into the chromatograph, record the chromatograms, and measure the responses for the major peaks. Calculate the quantity, in mg, of $C_{18}H_{24}O_2$ in the portion of Estradiol taken by the formula:

$$5C(R_U / R_S),$$

in which *C* is the concentration, in µg per mL, of USP Estradiol in the *Standard preparation;* and R_U and R_S are the peak responses obtained from the *Assay preparation* and the *Standard preparation*, respectively.

Estradiol Vaginal Cream

» Estradiol Vaginal Cream contains not less than [illegible] percent and not more than 110.0 percent of the labeled amount of $C_{18}H_{24}O_2$ in a suitable cream base.

Packaging and storage—Preserve in collapsible tubes or [illegible] containers.

USP Reference standards ⟨11⟩—*USP Estradiol RS. USP [illegible] RS.*

Identification—Transfer a portion of Vaginal Cream, equivalent to about 1 mg of estradiol, to a 150-mL beaker. Add 25 [illegible]

...onitrile, and gently heat to boiling. Boil for 45 seconds, and cool ...om temperature. Add 25 mL of water, and swirl. Filter with the ...of suction. Transfer the filtrate to a 125-mL separator, add 50 mL ...hloroform, and shake. Allow the layers to separate, drain the ...roform layer into a flask, and evaporate in a rotary evaporator to ...ess. Dissolve the residue in 2 mL of chloroform to obtain the test ...tion. Apply separately 50 µL of the test solution and 50 µL of a ...dard solution of USP Estradiol RS in chloroform containing ...out 0.5 mg per mL to a suitable thin-layer chromatographic plate ...e *Chromatography* ⟨621⟩) coated with a 0.25-mm layer of ...matographic silica gel mixture, and dry the applications with ...aid of a stream of nitrogen. Position the plate in a ...matographic chamber, and develop the chromatograms in a ...ent system consisting of a mixture of toluene and acetone (4 : 1) ...the solvent front has moved about three-fourths of the length of ...plate. Remove the plate from the developing chamber, mark the ...ent front, and allow the solvent to evaporate. Spray the plate with ...e mist of a mixture of sulfuric acid and methanol (1 : 1), then heat ...plate for 3 to 5 minutes at 90°. Observe the plate under visible ...t the R_F value and color of the principal spot obtained from the ...solution correspond to those obtained from the Standard solution.

...crobial limits ⟨61⟩—It meets the requirements of the tests for ...nce of *Staphylococcus aureus* and *Pseudomonas aeruginosa*.

...imum fill ⟨755⟩: meets the requirements.

...⟨791⟩: between 3.5 and 6.5.

...ssay—

...obile phase—Prepare a filtered and degassed mixture of ...onitrile and water (1:1). Make adjustments if necessary (see ...m *Suitability* under *Chromatography* ⟨621⟩).

...ternal standard solution—Dissolve a suitable quantity of ...rogesterone in acetonitrile to obtain a solution containing about ...g per mL. Use a freshly prepared solution.

...tandard preparation—Transfer about 10 mg of USP Estradiol RS ...about 7.5 mg of USP Estrone RS, both accurately weighed, to a ...-mL volumetric flask. Add 50.0 mL of *Internal standard ...tion* and 450 mL of acetonitrile, and mix. Dilute with water to ...me, and mix to obtain a solution having a known concentration ...out 10 µg of USP Estradiol RS per mL.

...ssay preparation—Transfer an accurately weighed portion of ...am, equivalent to about 0.5 mg of estradiol, to a 150-mL beaker. ...2.5 mL of *Internal standard solution*, 22.5 mL of acetonitrile, ...a few boiling chips. Cover with a watch glass, and heat gently ...the Cream melts, swirling occasionally. Heat to boiling for about ...econds. Allow to cool to room temperature, add 25.0 mL of ...er, and mix. Filter first through paper and then through a micro ...filter.

...romatographic system (see *Chromatography* ⟨621⟩)—The ...chromatograph is equipped with a 280-nm detector and a ...m × 30-cm column that contains packing L1. The flow rate is ...1 mL per minute. Chromatograph the *Standard preparation*, ...record the peak responses as directed under *Procedure:* the ...tion, R, between the analyte and estrone peaks is not less than ...and the relative standard deviation for replicate injections is not ...than 3.0%.

...ocedure—Separately inject equal volumes (about 50 µL) of the ...*dard preparation* and the *Assay preparation* into the chromat-..., record the chromatograms, and measure the responses for the ...peaks. The relative retention times are about 2.0 for the internal ...dard, 1.0 for estradiol, and 1.25 for estrone. Calculate the ..., in mg, of $C_{18}H_{24}O_2$ in the portion of Vaginal Cream taken by ...mula:

$$0.05C(R_U/R_S),$$

...in which C is the concentration, in µg per mL, of USP Estradiol RS in ...*dard preparation*, and R_U and R_S are the peak response ratios ...radiol and the internal standard obtained from the *Assay ...tion* and the *Standard preparation*, respectively.

Estradiol Pellets

» Estradiol Pellets are sterile pellets composed of Estradiol in compressed form, without the presence of any binder, diluent, or excipient. They contain not less than 97.0 percent and not more than 103.0 percent of $C_{18}H_{24}O_2$.

Packaging and storage—Preserve in tight containers, suitable for maintaining sterile contents, that hold 1 Pellet each.

USP Reference standards ⟨11⟩—*USP Estradiol RS.*

Solubility in chloroform—A solution of 25 mg of Pellets in 10 mL of chloroform is clear and practically free from insoluble residue.

Weight variation—Weigh 5 Pellets singly, and calculate the average weight. The average weight is between 95% and 105% of the labeled weight of $C_{18}H_{24}O_2$, and each Pellet weighs between 90% and 110% of the labeled weight of $C_{18}H_{24}O_2$.

Other requirements—Pellets meet the requirements under *Estradiol* and under *Sterility Tests* ⟨71⟩.

Assay—

Standard preparation—Prepare as directed in the *Assay* under *Estradiol Sterile Suspension.*

Assay preparation—Weigh and finely powder not less than 10 Pellets. Transfer a portion of the powder, equivalent to about 100 mg of estradiol, to a suitable container, dissolve in a sufficient quantity of a mixture of equal volumes of alcohol and chloroform to make 5.0 mL, and mix.

Procedure—Proceed as directed for *Procedure* in the *Assay* under *Estradiol Sterile Suspension.* Calculate the quantity, in mg, of $C_{18}H_{24}O_2$ in the portion of Pellets taken by the formula:

$$5C(A_U/A_S),$$

in which all terms are as defined therein.

Estradiol Injectable Suspension

» Estradiol Injectable Suspension is a sterile suspension of Estradiol in Water for Injection. It contains not less than 90.0 percent and not more than 110.0 percent of the labeled amount of $C_{18}H_{24}O_2$.

Packaging and storage—Preserve in single-dose or in multiple-dose containers, preferably of Type I glass.

USP Reference standards ⟨11⟩—*USP Endotoxin RS. USP Estradiol RS.*

Identification—Transfer a volume of well-mixed Injectable Suspension, equivalent to about 10 mg of estradiol, to a flask, render it acid to bromophenol blue TS with dilute hydrochloric acid (1 in 12), mix thoroughly, and place in an ice bath for 15 minutes. Filter the acidified suspension with suction through a sintered-glass funnel. Wash the crystals of estradiol so isolated with five successive 5-mL portions of water, and dry the funnel and contents at 105° to constant weight. The estradiol so obtained responds to *Identification* test *A* and meets the requirements of the test for *Melting range* under *Estradiol.*

Bacterial endotoxins ⟨85⟩—It contains not more than 250.0 USP Endotoxin Units per mg of estradiol.

Uniformity of dosage units ⟨905⟩: meets the requirements.

Other requirements—It meets the requirements under *Injections* ⟨1⟩.

Assay—

Standard preparation—Dissolve a suitable quantity of USP Estradiol RS, accurately weighed, in methanol, and dilute quantitatively, and stepwise if necessary, with methanol to obtain a solution having a known concentration of about 40 µg per mL.

Assay preparation—Transfer an accurately measured volume of well-mixed Injectable Suspension, equivalent to about 1 mg of estradiol, to a 100-mL beaker, and add water, if necessary, to obtain a

volume of about 5 mL. Add 6 g of purified siliceous earth, mix, and pack the mixture tightly into a 20- × 200-mm chromatographic tube containing in its base a pledget of fine glass wool. Dry-rinse the beaker with about 1 g of purified siliceous earth, add the rinsing to the packed column, and wipe out the beaker with a pledget of glass wool used to top the column. Elute the column with 50 mL of ether that previously has been saturated with water, and collect the eluate in a glass-stoppered, 125-mL conical flask. Evaporate with the aid of gentle heat and a current of air to dryness, add 25.0 mL of methanol to the residue, and mix.

Procedure—Transfer 1.0 mL each of the *Standard preparation* and the *Assay preparation* to separate glass-stoppered, 16- × 150-mm test tubes, and evaporate with the aid of gentle heat and a current of air to dryness. Using a suitable syringe, add 1.0 mL of iron-phenol TS to each tube and to a third, similar tube to provide the blank. Suspend the tubes in a vigorously boiling water bath, mixing them simultaneously after heating for 5 minutes. Remove the tubes after heating in the water bath for a total of 35 minutes, and immediately cool in an ice-water bath. Remove from the ice bath, add 10.0 mL of dilute sulfuric acid (1 in 3) to each tube, mix to obtain homogeneous solutions, and allow to reach room temperature. Concomitantly determine the absorbances of the solutions in 1-cm cells at the wavelength of maximum absorbance at about 520 nm, with a suitable spectrophotometer, against the blank. Calculate the quantity, in mg, of $C_{18}H_{24}O_2$ in each mL of the Injectable Suspension taken by the formula:

$$(0.025C/V)(A_U/A_S),$$

in which C is the concentration, in µg per mL, of USP Estradiol RS in the *Standard preparation*, V is the volume, in mL, of Injectable Suspension taken, and A_U and A_S are the absorbances of the solutions from the *Assay preparation* and the *Standard preparation*, respectively.

Estradiol Tablets

» Estradiol Tablets contain not less than 90.0 percent and not more than 115.0 percent of the labeled amount of $C_{18}H_{24}O_2$.

Packaging and storage—Preserve in tight, light-resistant containers.

USP Reference standards ⟨11⟩—*USP Estradiol RS.*

Identification—Place a quantity of finely powdered Tablets, equivalent to about 4 mg of estradiol, in a screw-capped, 20-mL vial. Add 10 mL of chloroform, and sonicate for 2 minutes. Filter through medium-porosity filter paper. Apply 20 µL each of this solution and a Standard solution of USP Estradiol RS in chloroform containing 0.4 mg per mL to a suitable thin-layer chromatographic plate (see *Chromatography* ⟨621⟩) coated with a 0.25-mm layer of chromatographic silica gel mixture. Allow the spots to dry, and develop the chromatogram in a lined chamber with a solvent system consisting of a mixture of toluene and acetone (4 : 1) until the solvent front has moved 10 cm beyond the starting line. Remove the plate from the developing chamber, mark the solvent front, and allow to air-dry. Spray the plate with a mixture of methanol and sulfuric acid (1 : 1), and heat at 100° for about 5 minutes: the principal spots obtained from the test solution and the Standard solution have the same color and R_F value.

Dissolution ⟨711⟩—
Medium: 0.3% sodium lauryl sulfate in water; 500 mL.
Apparatus 2: 100 rpm.
Time: 60 minutes.

Mobile phase—Prepare a suitable degassed and filtered solution of water and acetonitrile (55 : 45).

Standard solution—Prepare a solution of USP Estradiol RS in methanol having an accurately known concentration of about 0.02 mg per mL. Dilute aliquots of this solution with *Medium* to obtain a final solution having a concentration approximately equal to the expected concentration of drug in the *Medium*, assuming 100% dissolution.

Test solution—Use a filtered portion of the solution under test from the dissolution vessel.

Chromatographic system (see *Chromatography* ⟨621⟩)—The liquid chromatograph is equipped with a 205-nm detector and a 4.6-mm × 7.5-cm column that contains packing L1. The flow rate is about 1.5 mL per minute. Chromatograph replicate injections of the *Standard preparation*, and record the peak areas as directed in the *Procedure:* the tailing factor is not more than 2.0; and the relative standard deviation is not more than 2.0%.

Procedure—Separately inject equal volumes (about 100 µL) of the *Standard solution* and the *Test solution* into the chromatograph, record the chromatograms, and measure the areas for the major peaks. Calculate the quantity of $C_{18}H_{24}O_2$ dissolved by comparison of the peak areas obtained from the *Test solution* and the *Standard solution*.

Tolerances—Not less than 75% (*Q*) of the labeled amount of $C_{18}H_{24}O_2$ is dissolved in 60 minutes.

Uniformity of dosage units ⟨905⟩: meet the requirements.

Assay—

Mobile phase, Internal standard solution, Standard preparation, and *Chromatographic system*—Proceed as directed in the *Assay* under *Estradiol.*

Assay preparation—Weigh and finely powder not fewer than 10 Tablets. Transfer a portion of the powder, equivalent to about 8 mg of estradiol, to a 100-mL volumetric flask. Add 4 mL of water, and swirl. Add 10.0 mL of *Internal standard solution* and about 60 mL of methanol. Shake by mechanical means for 15 minutes, dilute with methanol to volume, mix, and allow the solids to settle. Filter a portion, discarding the first 10 mL of the filtrate. Mix 5.0 mL of the subsequent filtrate with 5.0 mL of methanol and 10.0 mL of water.

Procedure—Proceed as directed for *Procedure* in the *Assay* under *Estradiol.* Calculate the quantity, in mg, of $C_{18}H_{24}O_2$ in the portion of Tablets taken by the formula:

$$0.4C(R_U/R_S),$$

in which the terms are as defined therein.

Estradiol Cypionate

$C_{26}H_{36}O_3$ 396.57
Estra-1,3,5(10)-triene-3,17-diol, (17β)-, 17-cyclopentanepropanoate
Estradiol 17-cyclopentanepropionate [*313-06-4*].

» Estradiol Cypionate contains not less than 97.0 percent and not more than 103.0 percent of $C_{26}H_{36}O_3$, calculated on the dried basis.

Packaging and storage—Preserve in tight, light-resistant containers.

USP Reference standards ⟨11⟩—*USP Estradiol Cypionate RS.*

Identification—
A: *Infrared Absorption* ⟨197K⟩.
B: *Ultraviolet Absorption* ⟨197U⟩—
Solution: 100 µg per mL.
Medium: alcohol.
Absorptivities at 280 nm, calculated on the dried basis, do not differ by more than 3.0%.

Melting range ⟨741⟩: between 149° and 153°.

Specific rotation ⟨781S⟩: between +39° and +44°.
Test solution: 20 mg per mL, in dioxane.

Loss on drying ⟨731⟩—Dry it at 105° for 4 hours: it loses not more than 1.0% of its weight.

Residue on ignition ⟨281⟩: not more than 0.2%.

Assay—

Mobile phase—Dissolve 0.8 g of ammonium nitrate in 300 mL of water, add 700 mL of acetonitrile, and mix.

Internal standard solution—Prepare a solution of testosterone benzoate in tetrahydrofuran containing 2.0 mg per mL.

Standard preparation—Accurately weigh about 10 mg of USP Estradiol Cypionate RS, and transfer to a 10-mL volumetric flask. Add *Internal standard solution* to volume, and shake vigorously to dissolve.

Assay preparation—Using 10 mg of Estradiol Cypionate, accurately weighed, proceed as directed under *Standard preparation*

Procedure—Separately inject 10-µL aliquots of the *Assay preparation* and the *Standard preparation* into a suitable high-pressure liquid chromatograph fitted with a 280-nm detector, a 4-mm × 30-cm column containing packing L1 and operated at room temperature. The *Mobile phase* is maintained at a pressure and flow rate capable of giving the required resolution and a suitable elution time. In a suitable system, the resolution factor *R* (see *Chromatography* ⟨621⟩) is not less than 3.0 between the peaks for estradiol cypionate and the internal standard. Five replicate injections of the *Standard preparation* show a relative standard deviation that is not more than 1.5%. Calculate the quantity, in mg, of $C_{26}H_{36}O_3$ in the portion of Estradiol Cypionate taken by the formula:

$$10C(R_U / R_S),$$

in which *C* is the concentration, in mg per mL, of USP Estradiol Cypionate RS in the *Standard preparation;* and R_U and R_S are the ratios of the peak responses of the estradiol cypionate and internal standard peaks obtained from the *Assay preparation* and the *Standard preparation*, respectively.

Estradiol Cypionate Injection

» Estradiol Cypionate Injection is a sterile solution of Estradiol Cypionate in a suitable oil. It contains not less than 90.0 percent and not more than 110.0 percent of the labeled amount of $C_{26}H_{36}O_3$.

Packaging and storage—Preserve in single-dose or in multiple-dose, light-resistant containers, preferably of Type I glass.

USP Reference standards ⟨11⟩—*USP Estradiol Cypionate RS.*

Identification—Transfer a volume of Injection, equivalent to 5 mg of estradiol cypionate, to a glass-stoppered, 50-mL test tube, and add 30 mL of alcohol. Shake the mixture vigorously for 5 minutes, centrifuge until the two layers have separated, and transfer the alcohol layer, with the aid of a hypodermic syringe, to a 50-mL beaker. Evaporate on a steam bath to dryness, add 5 mL of potassium hydroxide solution (1 in 10), and heat on the steam bath for 15 minutes. Mix 50 mg of sulfanilic acid with 2 mL of 3 N hydrochloric acid, warm the mixture, then cool it in ice water, and slowly add, with agitation, 0.3 mL of sodium nitrite solution (1 in 10). Add this solution to the saponified estradiol cypionate: a red color is produced.

Other requirements—It meets the requirements under *Injections*

Assay—

Mobile Phase and *Internal standard solution*—Prepare as directed in the *Assay* under *Estradiol Cypionate*.

Standard preparation—Accurately weigh about 10 mg of USP Estradiol Cypionate RS, and transfer to a 100-mL volumetric flask. Add 10.0 mL of *Internal standard solution*, dilute with tetrahydrofuran to volume, and shake vigorously to dissolve.

Assay preparation—Using a "to contain" pipet, transfer an accurately measured volume, in mL, of Injection, equivalent to about 10 mg of estradiol cypionate, to a 100-mL volumetric flask. Rinse the pipet with small portions of tetrahydrofuran, collecting the rinsings in the volumetric flask. Add 10.0 mL of *Internal standard solution*, dilute with tetrahydrofuran to volume, and mix.

Procedure—Proceed as directed for *Procedure* in the *Assay* under *Estradiol Cypionate*. Calculate the quantity, in mg, of $C_{26}H_{36}O_3$ in mL of the Injection taken by the formula:

$$(100C / V)(R_U / R_S),$$

in which *C* is the concentration, in mg per mL, of USP Estradiol Cypionate RS in the *Standard preparation; V* is the volume, in mL, of Injection taken; and R_U and R_S are the ratios of the peak responses of estradiol cypionate and internal standard peaks obtained from the *Assay preparation* and the *Standard preparation*, respectively.

Estradiol Valerate

$C_{23}H_{32}O_3$ 356.50
Estra-1,3,5(10)-triene-3,17-diol(17β)-, 17-pentanoate.
Estradiol 17-valerate. [*979-32-8*].

» Estradiol Valerate contains not less than 98.0 percent and not more than 102.0 percent of $C_{23}H_{32}O_3$.

Packaging and storage—Preserve in tight, light-resistant containers.

USP Reference standards ⟨11⟩—*USP Estradiol Valerate RS.*

Identification, *Infrared Absorption* ⟨197K⟩.

Melting range, *Class Ia* ⟨741⟩: between 143° and 150°.

Specific rotation ⟨781S⟩: between +41° and +47°.

Test solution: 25 mg, uncorrected for moisture, per mL, in dioxane.

Water, *Method I* ⟨921⟩: not more than 0.1%.

Limit of estradiol—Apply 5 µL of a solution of Estradiol Valerate in acetone, containing 5 mg per mL, and 5 µL of a solution of estradiol in acetone, containing 50 µg per mL, about 2.5 cm from the lower edge of a thin-layer chromatographic plate (see *Chromatography* ⟨621⟩) coated with a 0.25-mm layer of chromatographic silica gel. Develop the chromatogram in a solvent system consisting of a mixture of cyclohexane and ethyl acetate (7 : 3) in an unlined chamber until the solvent front has moved about 15 cm above the point of application. Remove the plate, dry at 90° for 30 minutes, and spray the plate lightly with a 3 in 10 solution of methanol in sulfuric acid, prepared by cautiously adding sulfuric acid to 30 mL of methanol in a 100-mL volumetric flask, in an ice bath, to volume. Heat the plate at 90° for 30 minutes: any spot in the chromatogram of Estradiol Valerate close to the origin and corresponding to the estradiol spot is not larger nor more intense than that produced by the standard. (The limit is 1.0% of estradiol.)

Free acid—Neutralize 25 mL of alcohol, in a conical flask, with 0.01 N sodium hydroxide VS to a faint blue color, using bromothymol blue TS. Accurately weigh 500 mg of Estradiol Valerate, and dissolve it in the neutralized alcohol. Titrate rapidly with 0.01 N sodium hydroxide VS to a faint blue color. Each mL of 0.01 N sodium hydroxide is equivalent to 1.021 mg of valeric acid. The free acid content, expressed as valeric acid, does not exceed 0.5%.

Ordinary impurities ⟨466⟩—

Test solution: acetone.

Standard solution: acetone.

Eluant: a mixture of cyclohexane and ether (4 : 1).

Visualization: 5 followed by 1.

Assay—

Mobile phase—Dissolve 0.8 g of ammonium nitrate in 300 mL of water, add 700 mL of acetonitrile, and mix. Filter, and degas. Make adjustments if necessary (see *System Suitability* under *Chromatography* ⟨621⟩).

Internal standard solution—Prepare a solution of testosterone benzoate in tetrahydrofuran having a concentration of about 2.0 mg per mL.

Standard preparation—Dissolve an accurately weighed quantity of USP Estradiol Valerate RS in *Internal standard solution*, and dilute quantitatively with *Internal standard solution* to obtain a solution having a known concentration of about 1 mg of USP Estradiol Valerate RS per mL.

Assay preparation—Transfer about 25 mg of Estradiol Valerate, accurately weighed, to a 25-mL volumetric flask, add *Internal standard solution* to volume, and mix.

Chromatographic system (see *Chromatography* ⟨621⟩)—The liquid chromatograph is equipped with a 280-nm detector and a 4-mm × 30-cm column that contains packing L1. The flow rate is about 2 mL per minute. Chromatograph the *Standard preparation*, and record the peak responses as directed for *Procedure:* the relative retention times are about 1.2 for testosterone benzoate and 1.0 for estradiol valerate; the column efficiency determined from the analyte peak is not less than 1100 theoretical plates; the resolution, *R*, between the analyte and internal standard peaks is not less than 3.0; and the relative standard deviation for replicate injections is not more than 1.5%.

Procedure—Separately inject equal volumes (about 10 µL) of the *Standard preparation* and the *Assay preparation* into the chromatograph, record the chromatograms, and measure the responses for the major peaks. Calculate the quantity, in mg, of $C_{23}H_{32}O_3$ in the portion of Estradiol Valerate taken by the formula:

$$25C(R_U / R_S),$$

in which C is the concentration, in mg per mL, of USP Estradiol Valerate RS in the *Standard preparation;* and R_U and R_S are the peak response ratios obtained from the *Assay preparation* and the *Standard preparation*, respectively.

Estradiol Valerate Injection

» Estradiol Valerate Injection is a sterile solution of Estradiol Valerate in a suitable vegetable oil. It contains not less than 90.0 percent and not more than 115.0 percent of the labeled amount of $C_{23}H_{32}O_3$.

Packaging and storage—Preserve in single-dose or in multiple-dose, light-resistant containers, preferably of Type I or Type III glass.

USP Reference standards ⟨11⟩—*USP Estradiol Valerate RS.*

Identification—

Phenol reagent (*Folin-Ciocalteu reagent*)—Dissolve 100 g of sodium tungstate ($Na_2WO_4 \cdot 2H_2O$) and 25 g of sodium molybdate ($Na_2MoO_4 \cdot 2H_2O$) in 700 mL of water, in a 1500-mL flask connected by a standard taper joint to a reflux condenser. Add 50 mL of phosphoric acid and 100 mL of hydrochloric acid, and reflux gently for 10 hours. Cool, and add 150 g of lithium sulfate, 50 mL of water, and 4 to 6 drops of bromine. Boil the mixture without the condenser for 15 minutes to remove the excess bromine, cool, transfer to a 1-liter volumetric flask, dilute with water to volume, and filter: the filtrate is golden yellow in color, and has no greenish tint. Store the filtrate in a tight container in a refrigerator. Dilute 1 volume of the filtrate with 2 volumes of water prior to use as the *Phenol reagent.*

Procedure—Transfer 0.5 mL of Injection to a separator containing 10 mL of solvent hexane and 10 mL of 80% methanol. Shake the contents for 2 minutes, and allow the phases to separate. Add 1 mL of *Phenol reagent* and 3 mL of sodium carbonate solution (1 in 5) to 1 mL of the bottom layer, and mix: a blue color develops.

Limit of estradiol—Prepare a solution of estradiol in acetone containing 30.0% of the labeled concentration of the Injection, dilute 1.0 mL with the oil labeled as vehicle for the Injection to 10.0 mL, and mix. Apply 5 µL of Injection at a spot 2.5 cm from the bottom edge of and in the center of one section of a thin-layer chromatographic plate (see *Chromatography* ⟨621⟩) coated with a 0.25-mm layer of chromatographic silica gel, and apply 5 µL of the estradiol solution at the corresponding point in the other section of the plate. Allow the applications to be absorbed by the layer without air-drying, and proceed as directed in the test for *Limit of estradiol* under *Estradiol Valerate*, beginning with "Develop the chromatogram in a solvent system." (The limit of estradiol is 3.0%.)

Other requirements—It meets the requirements under *Injections* ⟨1⟩.

Assay—

Mobile phase, and *Chromatographic system*—Prepare as directed in the *Assay* under *Estradiol Valerate.*

Internal standard solution—Prepare a solution of testosterone benzoate in tetrahydrofuran having a concentration of about 8.0 mg per mL.

Standard preparation—Transfer about 20 mg of USP Estradiol Valerate RS, accurately weighed, to a 25-mL volumetric flask. Add 5.0 mL of the *Internal standard solution*, dilute with tetrahydrofuran to volume, and mix to obtain a solution having a known concentration of about 0.8 mg of USP Estradiol Valerate RS per mL.

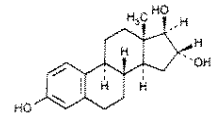
Assay preparation—Using a "to contain" pipet, transfer an accurately measured volume of Injection, equivalent to about 20 mg of estradiol valerate, to a 25-mL volumetric flask. Rinse the pipet with small portions of tetrahydrofuran, collecting the washings in the volumetric flask. Add 5.0 mL of *Internal standard solution*, dilute with tetrahydrofuran to volume, and mix.

Procedure—Proceed as directed for *Procedure* in the *Assay* under *Estradiol Valerate*. Calculate the quantity, in mg, of $C_{23}H_{32}O_3$ in each mL of the Injection taken by the formula:

$$25(C / V)(R_U / R_S),$$

in which C is the concentration, in mg per mL, of USP Estradiol Valerate RS in the *Standard preparation*, V is the volume, in mL, of Injection taken, and R_U and R_S are the peak response ratios obtained from the *Assay preparation* and the *Standard preparation*, respectively.

Estriol



$C_{18}H_{24}O_3$ 288.38
Estra-1,3,5(10)-triene-3,16,17-triol, (16α,17β)-.
Estriol [*50-27-1*].

» Estriol contains not less than 97.0 percent and not more than 102.0 percent of $C_{18}H_{24}O_3$, calculated on the dried basis.

Packaging and storage—Preserve in tight containers.

USP Reference standards ⟨11⟩—*USP Estriol RS.*

Completeness of solution—Dissolve 500 mg in 10 mL of pyridine: the solution is clear and free from undissolved solid.

Identification—

A: *Infrared Absorption* ⟨197K⟩.

B: *Ultraviolet Absorption* ⟨197U⟩—

Solution: 100 µg per mL.

Medium: alcohol.

Specific rotation ⟨781S⟩: between +54° and +62°.

Test solution: 4 mg per mL, in dioxane.

Loss on drying ⟨731⟩—Dry it at 105° for 3 hours: it loses not more than 0.5% of its weight.

Residue on ignition ⟨281⟩: not more than 0.1%.

Chromatographic purity—

Test preparation—Prepare a solution of Estriol in a mixture of dioxane and water (9 : 1) to obtain a solution containing 20.0 mg per mL.

Standard solution and *Standard dilutions*—Prepare a solution of USP Estriol RS in a mixture of dioxane and water (9 : 1) to obtain a solution containing 20 mg per mL (*Standard solution*). Prepare a series of dilutions of the *Standard solution* in a mixture of dioxane and water (9 : 1) to obtain solutions containing 0.40, 0.20, 0.10, and 0.05 mg per mL (*Standard dilutions*).

Chromatographic chamber—Line a suitable chamber (see *Chromatography* ⟨621⟩) with absorbent paper, and pour into the chamber 200 mL of developing solvent, prepared by mixing, just prior to use, 90 mL of chloroform, 5 mL of methanol, 5 mL of acetone, and 5 mL of acetic acid. Equilibrate the chamber for 15 minutes before using.

Procedure—Apply 5-µL volumes of the *Test preparation*, *Standard solution*, and each of the four *Standard dilutions* at equidistant points along a line 2.5 cm from one edge of a 20- × 20-cm thin-layer chromatographic plate (see *Chromatography* ⟨621⟩) coated with a 0.25-mm layer of chromatographic silica gel mixture. Place the plate in the *Chromatographic chamber*, seal the chamber, and allow the chromatogram to develop until the solvent front has moved 15 cm above the line of application. Remove the plate, and allow the solvent to evaporate. Spray the plate with a mixture of methanol and sulfuric acid (7 : 3), then heat the plate at 100° for 5 minutes. The lane of the *Test preparation* exhibits its principal spot at the same R_F value as the principal spot of the *Standard solution*. Any spots other than the principal spot are observed in the lane of the

Assay ...*aration*, estimate the concentration of each by comparison with ...*Standard dilutions*. The spots from the 0.40-, 0.20-, 0.10-, and ...-mg-per-mL dilutions are equivalent to 2.0%, 1.0%, 0.5%, and ...% of impurities, respectively. The requirements of the test are ...if the sum of impurities in the *Test preparation* is not greater than ...%.

...SP Estriol RS, accurately weighed, in ...ethanol to make 100.0 mL, and mix. Dilute 10.0 mL of this solution ...with alcohol to 100.0 mL. Similarly, dissolve a suitable quantity of ...USP Estriol RS, accurately weighed, in alcohol to obtain a Standard ...solution having a known concentration of about 50 µg per mL. ...Concomitantly determine the absorbances of both solutions in 1-cm ...cells at the wavelength of maximum absorbance at about 281 nm. ...Calculate the quantity, in mg, of $C_{18}H_{24}O_3$ in the portion of Estriol ...taken by the formula:

$$C(A_U / A_S),$$

...in which C is the concentration, in µg per mL, of USP Estriol RS in ...the Standard solution, and A_U and A_S are the absorbances of the ...solution of Estriol and the Standard solution, respectively.

Conjugated Estrogens

Conjugated Estrogens is a mixture of sodium estrone ...sulfate and sodium equilin sulfate, derived wholly or in ...part from equine urine or synthetically from Estrone and ...equilin. It contains other conjugated estrogenic sub...stances of the type excreted by pregnant mares. It is a ...dispersion of the estrogenic substances on a suitable ...powdered diluent.

Conjugated Estrogens contains not less than 52.5 ...percent and not more than 61.5 percent of sodium ...estrone sulfate and not less than 22.5 percent and not ...more than 30.5 percent of sodium equilin sulfate, and the ...total of sodium estrone sulfate and sodium equilin sulfate ...is not less than 79.5 percent and not more than 88.0 ...percent of the labeled content of Conjugated Estrogens. ...Conjugated Estrogens contains as concomitant compo...nents as sodium sulfate conjugates not less than 13.5 ...percent and not more than 19.5 percent of 17α-...dihydroequilin, not less than 2.5 percent and not more ...than 9.5 percent of 17α-estradiol, and not less than 0.5 ...percent and not more than 4.0 percent of 17β-...dihydroequilin, of the labeled content of Conjugated ...Estrogens.

Packaging and storage—Preserve in well-closed containers. Store at ..., excursions permitted between 15° and 30°.

Labeling—Label it to state the content of Conjugated Estrogens on a ...weight-to-weight basis.

USP Reference standards ⟨11⟩—*USP 17α-Dihydroequilin RS. USP ...Equilin RS. USP Estradiol RS. USP Estrone RS.*

Identification—The following results are obtained with respect to ...*Assay preparation* treated as directed for *Procedure* in the *Assay*.

A: The chromatogram exhibits peaks for 17α-dihydroequilin, ...estrone, and equilin at relative retention times corresponding to those ...exhibited in the chromatogram of the *Standard preparation*.

B: The chromatogram of Conjugated Estrogens exhibits addi...tional peaks or shoulders, corresponding to 17α-estradiol and 17β-...dihydroequilin at retention times of about 0.24 and 0.35, respectively, ...relative to that of 3-*O*-methylestrone.

Content of 17α-dihydroequilin, 17β-dihydroequilin, and 17α-estradiol (concomitant components)—*Internal standard solution, Stock solution, pH 5.2 Acetate buffer, System suitability solution, Standard preparation,* and *Chromatographic system*—Proceed as directed in the *Assay*.

Test preparation—Prepare as directed for *Assay preparation* in the *Assay*.

Procedure—Separately inject equal volumes (about 1 µL) of the *Standard preparation* and the *Test preparation* into the chromatograph, record the chromatograms, and identify the peaks due to 17α-estradiol, 17α-dihydroequilin, and 17β-dihydroequilin in the chromatogram of the *Test preparation*. The relative retention times relative to 17α-dihydroequilin are about 0.82, 1.00, and 1.11 for 17α-estradiol, 17α-dihydroequilin, and 17β-dihydroequilin, respectively. Separately calculate the quantities, in mg, of 17α-estradiol, 17α-dihydroequilin, and 17β-dihydroequilin as their sodium sulfate salts in the portion of Conjugated Estrogens taken by the formula:

$$0.005(1.381C_S)(R_U / R_S),$$

in which C_S is the concentration, in µg per mL, of USP 17α-Dihydroequilin RS in the *Stock solution*; R_U is the ratio of the peak response of the appropriate analyte to that of the internal standard obtained from the *Test preparation*; and R_S is the ratio of the peak response of 17α-dihydroequilin to that of the internal standard obtained from the *Standard preparation*.

Limits of 17α-dihydroequilenin, 17β-dihydroequilenin, and equilenin (signal impurities)—*Internal standard solution, Stock solution, pH 5.2 Acetate buffer, System suitability solution, Standard preparation,* and *Chromatographic system*—Proceed as directed in the *Assay*.

Test preparation—Prepare as directed for *Assay preparation* in the *Assay*.

Procedure—Separately inject equal volumes (about 1 µL) of the *Standard preparation* and the *Test preparation* into the chromatograph, record the chromatograms, and identify any peaks due to dihydroequilenin, 17β-dihydroequilenin, 3-*O*-methylestrone, and equilenin in the chromatogram of the *Assay preparation*. The relative retention times for these peaks are about 0.56, 0.64, 1.0, and 1.3, respectively. Separately calculate the quantities, in mg, of 17α-dihydroequilenin, 17β-dihydroequilenin, and equilenin as their sodium sulfate salts in the portion of Conjugated Estrogens taken by the formula:

$$0.005(1.381C_S)(R_U / R_S),$$

in which C_S is the concentration, in µg per mL, of USP Estrone RS in the *Stock solution*; R_U is the ratio of the peak response of the appropriate analyte to that of the internal standard obtained from the *Test preparation*; and R_S is the ratio of the peak response of estrone to that of the internal standard obtained from the *Standard preparation*. The limits of 17α-dihydroequilenin, 17β-dihydroequilenin, and equilenin as their sodium sulfate salts are not more than 3.25%, 2.75%, and 5.5%, respectively, of the labeled content of Conjugated Estrogens.

Limits of 17β-estradiol and $\Delta^{8,9}$-dehydroestrone—*Internal standard solution, Stock solution, pH 5.2 Acetate buffer, System suitability solution, Standard preparation,* and *Chromatographic system*—Proceed as directed in the *Assay*.

Test preparation—Prepare as directed for *Assay preparation* in the *Assay*.

Procedure—Separately inject equal volumes (about 1 µL) of the *Standard preparation* and the *Test preparation* into the chromatograph, record the chromatograms, and identify any peaks due to 17β-estradiol, 3-*O*-methylestrone, and $\Delta^{8,9}$-dehydroestrone in the chromatogram of the *Test preparation*. The relative retention times of these peaks are about 0.29, 1.0, and 0.9, respectively, relative to the internal standard. Separately calculate the quantities, in mg, of 17β-estradiol and $\Delta^{8,9}$-dehydroestrone as their sodium sulfate salts in the portion of Conjugated Estrogens taken by the formula:

$$0.005(1.381C_S)(R_U / R_S),$$

in which C_S is the concentration, in µg per mL, of USP Estrone RS in the *Stock solution*; R_U is the ratio of the peak response of the appropriate analyte to that of the internal standard obtained from the *Test preparation*; and R_S is the ratio of the peak response of estrone to that of the internal standard obtained from the *Standard preparation*. The limits of 17β-estradiol and $\Delta^{8,9}$-dehydroestrone as their sodium sulfate salts are not more than 2.25% and 6.25%, respectively, of the labeled content of Conjugated Estrogens.

Limit of estrone, equilin, and 17α-dihydroequilin (free steroids)—*Internal standard solution, pH 5.2 Acetate buffer, Stock solution,* and *System suitability solution*—Proceed as directed in the *Assay*.

Free steroids standard solution—Dilute the *Stock solution* tenfold. Pipet 1.0 mL of the resulting solution and 1.0 mL of the *Internal standard solution* into a suitable centrifuge tube fitted with a tight screw cap or stopper. Proceed as directed for *Standard preparation* in the *Assay,* beginning with "Evaporate the mixture."

Test solution—Proceed as directed for *Assay preparation* in the *Assay* with the following exceptions: do not add the sulfatase enzyme preparation, and transfer 6.0 mL of the filtrate instead of 3.0 mL in the preparation of the test specimen. Prepare a reagent blank in the same manner.

Chromatographic system—Proceed as directed in the *Assay* with the additional requirement that the relative standard deviation for the ratio of the peak response of estrone to that of the internal standard in the *Free steroids standard solution* is not greater than 5.5%, on the basis of not less than two replicate injections.

Procedure—Separately inject equal volumes (about 1 µL) of the *Free steroids standard solution* and the *Test solution* into the chromatograph, record the chromatograms, and measure the peak responses. Calculate the ratio, R_U, of the combined peak areas of estrone, equilin, and 17α-dihydroequilin relative to the area of the internal standard in the *Test solution,* correcting for any reagent blank peaks. The ratio, R_U / R_S, where R_S is the peak response ratio of estrone to that of the internal standard obtained from the *Free steroids standard solution,* is not more than 0.65 (1.3% of free steroids).

Organic volatile impurities, *Method V* ⟨467⟩: meets the requirements.

Solvent—Use dimethyl sulfoxide.

Assay—

Internal standard solution—Prepare a solution of 3-*O*-methylestrone in methanol containing about 150 µg per mL.

Stock solution—Using accurately weighed quantities of USP Estrone RS, USP Equilin RS, and USP 17α-Dihydroequilin RS, prepare, by quantitative and stepwise dilution, a solution in alcohol having known concentrations of about 160, 70, and 50 µg per mL, respectively.

pH 5.2 Acetate buffer—Mix 79 mL of sodium acetate TS with 21 mL of 1 N acetic acid, dilute with water to 500 mL, and mix. Adjust to a pH of 5.2 ± 0.1 by the addition of 1 N acetic acid or sodium acetate TS, if necessary.

System suitability solution—Dissolve a quantity of USP Estradiol RS (17β-estradiol) in alcohol to obtain a solution containing about 2 µg per mL. Pipet 1.0 mL of this solution, 1.0 mL of *Stock solution,* and 1.0 mL of *Internal standard solution* into a centrifuge tube fitted with a tight screw cap or stopper. Proceed as directed for *Standard preparation,* beginning with "Evaporate the mixture."

Standard preparation—Pipet 1.0 mL of the *Stock solution* and 1.0 mL of *Internal standard solution* into a suitable centrifuge tube fitted with a tight screw cap or stopper. Evaporate the mixture with the aid of a stream of nitrogen to dryness, maintaining the temperature below 50°. To the dry residue add 15 µL of dried pyridine and 65 µL of bis(trimethylsilyl)trifluoroacetamide containing 1% trimethylchlorosilane. Immediately cover the tube tightly, mix, and allow to stand for 15 minutes. Add 0.5 mL of toluene, and mix.

Assay preparation—Transfer an accurately weighed quantity of Conjugated Estrogens, equivalent to about 2 mg of total conjugated estrogens, to a 50-mL centrifuge tube, fitted with a polytef-lined screw cap, containing 15 mL of *pH 5.2 Acetate buffer* and 1 g of barium chloride. Cap the tube tightly, and shake for 30 minutes. If necessary, adjust the solution with 1 N acetic acid or sodium acetate to a pH of 5.0 ± 0.5. Place in a sonic bath for 30 seconds, then shake for an additional 30 minutes. Add a suitable sulfatase enzyme preparation equivalent to 2500 Units, and shake for 20 minutes in a water bath maintained at 50°. Add 15.0 mL of ethylene dichloride to the warm mixture, cap the tube again, and shake by mechanical means for 15 minutes. Centrifuge for 10 minutes or until the lower layer is clear. Transfer as much of the organic phase as possible, and dry by filtering rapidly through a filter consisting of a pledget of dry glass wool and about 5 g of anhydrous sodium sulfate in a small funnel. Protect from loss by evaporation. Transfer 3.0 mL of the solution to a suitable centrifuge tube fitted with a tight screw cap or stopper. Add 1.0 mL of *Internal standard solution.* Proceed as directed under *Standard preparation,* beginning with "Evaporate the mixture."

Chromatographic system (see *Chromatography* ⟨621⟩)—The gas chromatograph is equipped with a flame-ionization detector maintained at a temperature of 260°, a 0.25-mm × 15-m fused silica capillary column bonded with a 0.25-µm layer of phase G19, and a split injection system. The column temperature is maintained at 2[illegible] and the injection port at 260°. The carrier gas is hydrogen flowing [illegible] the rate of 2 mL per minute, and the split flow rate is 40 to 60 mL [illegible] minute. Inject about 1 µL of the *System suitability solution* into [illegible] gas chromatograph. Adjust the operating conditions as necessary [illegible] maintain the elution time of the 3-*O*-methylestrone peak at betw[illegible] 17 and 25 minutes. The relative retention times are about 0.29, 0.[illegible] 0.80, 0.87, and 1.00 for 17β-estradiol, 17α-dihydroequilin, estr[illegible] equilin, and 3-*O*-methylestrone, respectively. The tailing factor [illegible] the estrone peak is not more than 1.3; the resolution, *R*, betw[illegible] estrone and equilin is not less than 1.2; and the relative stand[illegible] deviation of the estrone peak ratios is not greater than 2.0% for [illegible] fewer than four injections of the *Standard preparation.*

Procedure—Separately inject equal volumes (about 1 µL) of [illegible] *Standard preparation* and the *Assay preparation* into the chroma[illegible] ograph, record the chromatograms, and measure the responses for [illegible] major peaks. Separately calculate the quantities, in mg, of sodium [illegible] estrone sulfate and sodium equilin sulfate in the portion [illegible] Conjugated Estrogens taken by the formula:

$$0.005(1.381C_S)(R_U / R_S),$$

in which 1.381 is the factor converting free estrogen to the conjug[illegible] sodium salt; C_S is the concentration, in µg per mL, of USP Estrone [illegible] or USP Equilin RS in the *Stock solution;* and R_U and R_S are the ra[illegible] of the peak response of the appropriate analyte to that of the intern[illegible] standard obtained from the *Assay preparation* and the *Standa[illegible] preparation,* respectively.

Conjugated Estrogens Tablets

» Conjugated Estrogens Tablets contain not less th[illegible] 73.0 percent and not more than 95.0 percent of th[illegible] labeled amount of conjugated estrogens as the total [illegible] sodium estrone sulfate and sodium equilin sulfate. Th[illegible] ratio of sodium equilin sulfate to sodium estrone sulfa[illegible] in the Tablets is not less than 0.35 and not more th[illegible] 0.65.

Packaging and storage—Preserve in well-closed containers.

Labeling—The labeling indicates the Tablet strength and states [illegible] which in vitro *Drug Release Test* the product complies.

USP Reference standards ⟨11⟩—*USP 17α-Dihydroequilin RS. USP Equilin RS. USP Estrone RS. USP Testosterone RS.*

Identification—Tablets respond to the *Identification* tests [illegible] *Conjugated Estrogens.*

Change to read:

Drug release ⟨724⟩—Proceed as directed for *Extended-Rele[illegible] Articles—General Drug Release Standard.*

TEST 1 (for products labeled as 0.3-,▲0.45-,▲*USP28* and 0.625-[illegible] tablets)—If the product complies with this test, the labeling indicat[illegible] that it meets USP *Drug Release Test 1.*

Medium: water; 900 mL.

Apparatus 2: 50 rpm.

Mobile phase—Prepare a filtered and degassed mixture of 0.02[illegible] monobasic potassium phosphate and acetonitrile (3:1). M[illegible] adjustments if necessary (see *System Suitability* under *Chroma[illegible] raphy* ⟨621⟩).

Standard solution—Transfer 10 Tablets to a 1000-mL volume[illegible] flask, dilute with water to volume, and stir vigorously by mechan[illegible] means for at least 3 hours. Pipet a filtered 100-mL aliquot of [illegible] solution into a 900-mL volumetric flask, and dilute with wate[illegible] volume.

Test solution—Filter a portion of the solution under test. [NOTE—[illegible] is recommended that the filters selected be tested for binding affini[illegible]

Chromatographic system—The liquid chromatograph is equip[illegible] with a 205-nm detector and a 4.6-mm × 3.0-cm column that cont[illegible] 3-µm packing L1. The flow rate is about 1.5 mL per minu[illegible] Chromatograph replicate injections of the *Standard solution* [illegible] record the responses as directed for *Procedure:* the relative reten[illegible] times are about 0.9 for equilin sulfate and 1.0 for estrone sulfate[illegible]

estrone sulfate peak being the last major peak in the chromatogram; the resolution, R, between equilin sulfate and estrone sulfate is not less than 1.5; and the relative standard deviation for the estrone sulfate peak is not more than 1.5%. [NOTE—If estrone is present it may be retained on the column for a period longer than 50 minutes and interfere in later chromatographic runs.]

Procedure—Separately inject equal volumes (between 20 and 200 µL) of the *Standard solution* and the *Test solution* into the chromatograph, record the chromatograms, and measure the responses for the estrone sulfate peaks. Calculate the percentage of estrone sodium sulfate released by the formula:

$$100(r_U / r_S),$$

in which r_U and r_S are the peak responses obtained from the *Test solution* and the *Standard solution*, respectively.

Times and *tolerances*—The percentages of estrone sodium sulfate dissolved at the times specified conform to *Acceptance Table 1*.

Time (hours)	Amount dissolved
2	between 19% and 49%
5	between 66% and 96%
8	not less than 80%

TEST 2 (for products labeled as 0.9-mg tablets)—If the product complies with this test, the labeling indicates that it meets USP *Drug Release Test 2*.

Medium, Apparatus, Mobile phase, Standard solution, Test solution, Chromatographic system, and *Procedure*—Proceed as directed for *Test 1*.

Times and *tolerances*—The percentages of estrone sodium sulfate dissolved at the times specified conform to *Acceptance Table 1*.

Time (hours)	Amount dissolved
2	between 12% and 37%
5	between 57% and 85%
8	not less than 80%

TEST 3 (for products labeled as 1.25- and 2.50-mg tablets)—If the product complies with this test, the labeling indicates that it meets USP *Drug Release Test 3*.

Medium, Apparatus, Mobile phase, Standard solution, Test solution, Chromatographic system, and *Procedure*—Proceed as directed for *Test 1*.

Times and *tolerances*—The percentages of estrone sodium sulfate dissolved at the times specified conform to *Acceptance Table 1*.

Time (hours)	Amount dissolved
2	between 3% and 22%
5	between 37% and 67%
8	between 66% and 96%
12	not less than 80%

Uniformity of dosage units—Assay 10 individual Tablets as directed in the *Assay*, and calculate the average content of conjugated estrogens, as the average of the total contents of sodium estrone sulfate and sodium equilin sulfate, in the 10 Tablets. The requirements are met if the content of each of the Tablets is not less than 85.0% and not more than 115.0% of the average content of conjugated estrogens. If the content of not more than 2 Tablets falls outside the range of 85.0% to 115.0% of the average content but not outside the range of 75.0% to 125.0%, assay an additional 20 Tablets. The requirements are met if the content of not more than 2 of the 30 Tablets falls outside the limits of 85.0% and 115.0% of that average, and no unit is outside the range of 75.0% to 125.0% of the average content.

Assay—

Internal standard solution, Stock solution, pH 5.2 Acetate buffer, System suitability solution, Standard preparation, and *Chromatographic system*—Proceed as directed in the *Assay* under *Conjugated Estrogens*.

Assay preparation—If the Tablets are sugar-coated, carefully remove the color and sugar coatings with water, leaving the shellac coating intact, and dry under nitrogen. Weigh and finely powder not fewer than 20 of the Tablets. Transfer an accurately weighed quantity of the powder, equivalent to about 2 mg of total conjugated estrogens, to a 50-mL centrifuge tube fitted with a polytef-lined screw-cap and containing 15 mL of *pH 5.2 Acetate buffer* and 1 g of barium chloride. Proceed as directed in the *Assay preparation* under *Conjugated Estrogens*, beginning with "Cap the tube tightly."

Procedure—Separately inject equal volumes (about 1 µL) of the *Standard preparation* and the *Assay preparation* into the chromatograph, record the chromatograms, and measure the responses for the major peaks. Separately calculate the quantities, in mg, of sodium estrone sulfate and sodium equilin sulfate in the portion of Tablets taken by the formula:

$$0.005(1.381C_S)(R_U / R_S),$$

in which 1.381 is the factor converting free estrogen to the conjugate sodium salt; C_S is the concentration, in µg per mL, of USP Estrone RS or USP Equilin RS in the *Stock solution;* and R_U and R_S are the ratios of the peak response of the appropriate analyte to that of the internal standard obtained from the *Assay preparation* and the *Standard preparation,* respectively.

Esterified Estrogens

» Esterified Estrogens is a mixture of the sodium salts of the sulfate esters of the estrogenic substances, principally estrone. It is a dispersion of the estrogenic substances on a suitable powdered diluent. The content of total esterified estrogens is not less than 90.0 percent and not more than 110.0 percent of the labeled amount.

Esterified Estrogens contains not less than 75.0 percent and not more than 85.0 percent of sodium estrone sulfate, and not less than 6.0 percent and not more than 15.0 percent of sodium equilin sulfate, in such proportion that the total of these two components is not less than 90.0 percent, of the labeled amount of esterified estrogens.

Packaging and storage—Preserve in tight containers.

Labeling—Label it to state the content of Esterified Estrogens on a weight-to-weight basis.

USP Reference standards ⟨11⟩—*USP Equilin RS. USP Estrone RS. USP Estradiol RS.*

Identification—It responds to *Identification* test *A* under *Conjugated Estrogens*.

Free steroids—Proceed with Esterified Estrogens as directed in the test for *Limit of estrone, equilin, and 17α-dihydroequilin* (free steroids) under *Conjugated Estrogens*. The limit is 3.0% of free steroids.

Organic volatile impurities, *Method V* ⟨467⟩: meets the requirements.

Solvent—Use dimethyl sulfoxide.

Assay—

Internal standard solution, Stock solution, Acetate buffer, pH 5.2, System suitability solution, Standard preparation, and *Chromatographic system*—Proceed as directed in the *Assay* under *Conjugated Estrogens*.

Assay preparation—Using an accurately weighed quantity of Esterified Estrogens, equivalent to about 2 mg of total esterified estrogens, proceed as directed for *Assay preparation* in the *Assay* under *Conjugated Estrogens*.

Procedure—Proceed as directed in the *Assay* under *Conjugated Estrogens*. Calculate the quantity, in mg, of each sodium estrogen sulfate (estrone and equilin) in the portion of Esterified Estrogens taken by the formula:

$$(0.005)(F)C_U(R_U / R_S),$$

in which F is the factor converting free estrogen to the conjugate sodium salt, the factor being 1.377 for estrone and 1.380 for equilin; C_U is the concentration, in µg per mL, of USP Estrone RS or USP Equilin RS, as appropriate, in the alcohol solution; and R_U and R_S are

the ratios of the estrone or equilin peak areas to the 3-*O*-methylestrone peak areas obtained from the *Assay preparation* and the *Standard preparation*, respectively.

Esterified Estrogens Tablets

» Esterified Estrogens Tablets contain not less than 90.0 percent and not more than 115.0 percent of the labeled amount of esterified estrogens as the total of sodium estrone sulfate and sodium equilin sulfate. The ratio of sodium equilin sulfate to sodium estrone sulfate is not less than 0.071 and not more than 0.20.

Packaging and storage—Preserve in well-closed containers.

USP Reference standards ⟨11⟩—*USP Equilin RS. USP Estrone RS. USP Testosterone RS.*

Identification—Tablets respond to the *Identification* test under *Esterified Estrogens.*

Disintegration ⟨701⟩—

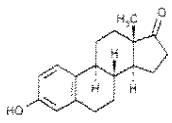
Simulated intestinal fluid—Dissolve 6.8 g of monobasic potassium phosphate in 250 mL of water, mix, and add 190 mL of 0.2 N sodium hydroxide and 400 mL of water. Add 10.0 g of pancreatin, mix, and adjust the resulting solution with 0.2 N sodium hydroxide to a pH of 7.5 ± 0.1. Dilute with water to 1000 mL.

Procedure—Place 1 Tablet in each of the six tubes of the basket, and immerse the basket in water at 25 ± 0.5° for 5 minutes to remove the coating. Add a disk to each tube, and operate the apparatus using simulated gastric fluid TS, maintained at 37 ± 2°, as the immersion fluid. After 30 minutes in simulated gastric fluid TS, lift the basket from the fluid, and observe the Tablets: all the Tablets have disintegrated. If all the Tablets have not disintegrated completely, substitute *Simulated intestinal fluid*, maintained at 37 ± 2°, as the immersion fluid, and continue the test so that the total period of time, including previous exposure to water and simulated gastric fluid TS, does not exceed 90 minutes.

Uniformity of dosage units—Assay 10 individual Tablets as directed in the *Assay*, and calculate the average content of esterified estrogens, as the average of the total contents of sodium estrone sulfate and sodium equilin sulfate, in the 10 Tablets. The requirements are met if the content of each of the Tablets is not less than 85.0 percent and not more than 115.0 percent of the average content of esterified estrogens. If the content of not more than 2 Tablets falls outside the range of 85.0 percent to 115.0 percent of the average content but not outside the range of 75.0 percent to 125.0 percent, assay an additional 20 Tablets. The requirements are met if the content of not more than 2 of the 30 Tablets falls outside the limits of 85.0 percent and 115.0 percent of the average, and no unit is outside the range of 75.0 percent to 125.0 percent of the average content.

Assay—Weigh and finely powder not less than 20 Tablets. Using a suitable portion of the powder, proceed as directed in the *Assay* under *Conjugated Estrogens.*

Estrone



$C_{18}H_{22}O_2$ 270.37
Estra-1,3,5(10)-trien-17-one, 3-hydroxy-.
3-Hydroxyestra-1,3,5(10)-trien-17-one [*53-16-7*].

» Estrone contains not less than 97.0 percent and not more than 103.0 percent of $C_{18}H_{22}O_2$, calculated on the dried basis.

Packaging and storage—Preserve in tight, light-resistant containers. Store at 25°, excursions permitted between 15° and 30°.

USP Reference standards ⟨11⟩—*USP Estrone RS.*

Clarity of solution—Add 100 mg to 100 mL of 1 N sodium hydroxide in a 125-mL conical flask, heat on a steam bath until solution is complete, then cool, and transfer to a 100-mL colorless comparison tube: the solution is clear.

Identification—

A: *Infrared Absorption* ⟨197K⟩.

B: *Ultraviolet Absorption* ⟨197U⟩—

Solution: 50 µg per mL.

Medium: alcohol, heated on a steam bath and cooled to room temperature.

Specific rotation ⟨781S⟩: between +158° and +165°.

Test solution: 10 mg, previously dried, per mL, in dioxane.

Loss on drying ⟨731⟩—Dry it at 105° for 3 hours: it loses not more than 0.5% of its weight.

Residue on ignition ⟨281⟩: not more than 0.5%.

Limit of equilenin and equilin—Dissolve 10 mg in sufficient alcohol to make 50 mL. Transfer 5 mL of the solution to a small beaker. Add 5 mL of a buffer solution prepared by dissolving 2 mL of glacial acetic acid and 13.3 g of anhydrous sodium acetate in water to make 100 mL, warm to about 50°, and add 1 mL of a freshly prepared 1 in 200 solution of 2,6-dibromoquinone-chlorimide in alcohol. Mix, and allow to stand for 30 minutes. Transfer the solution to a small separator, add 10 mL of chloroform and 20 mL of 1 N sodium hydroxide, and shake vigorously for 2 minutes. Separate the chloroform layer, and filter rapidly through a dry filter paper into a dry test tube, discarding the first 2 mL of the filtrate. Viewed transversely against a white background, the chloroform filtrate shows no more red color than that produced by similarly treating 5 mL of an alcohol solution containing 20 µg of equilenin.

Ordinary impurities ⟨466⟩—

Test solution: acetone.

Standard solution: acetone.

Eluant: a mixture of chloroform and acetone (9:1), in a nonequilibrated chamber.

Visualization: 5.

Assay—

Mobile phase—Prepare a filtered and degassed mixture of acetonitrile and 0.05 M monobasic potassium phosphate (1:1). Make adjustments if necessary (see *System Suitability* under *Chromatography* ⟨621⟩).

Standard preparation—Transfer about 20 mg of USP Estrone RS, accurately weighed, to a 100-mL volumetric flask, add methanol to volume, and mix. If necessary, sonicate to aid solution. Transfer 5 mL of this solution to a 25-mL volumetric flask, dilute with *Mobile phase* to volume, and mix to obtain a *Standard preparation* having a known concentration of about 40 µg of USP Estrone RS per mL.

Assay preparation—Transfer about 20 mg of Estrone, accurately weighed, to a 100-mL volumetric flask, add methanol to volume, and mix. If necessary, sonicate to aid solution. Transfer 5.0 mL of this solution to a 25-mL volumetric flask, dilute with *Mobile phase* to volume, and mix.

Chromatographic system (see *Chromatography* ⟨621⟩)—The liquid chromatograph is equipped with a 280-nm detector and a 4-mm × 15-cm column that contains 5-µm packing L1. The flow rate is about 1 mL per minute. Chromatograph the *Standard preparation*, and record the peak responses as directed for *Procedure:* the column efficiency determined from the analyte peak is not less than 1500 theoretical plates, the tailing factor for the analyte peak is not more than 2.0, and the relative standard deviation for replicate injections is not more than 2.0%.

Procedure—Separately inject equal volumes (about 50 µL) of the *Standard preparation* and the *Assay preparation* into the chromatograph, record the chromatograms, and measure the responses for the

major peaks. Calculate the quantity, in mg, of $C_{18}H_{22}O_2$ in the portion of Estrone taken by the formula:

$$0.5C(r_U / r_S),$$

in which C is the concentration, in µg per mL, of USP Estrone RS in the *Standard preparation;* and r_U and r_S are the peak responses obtained from the *Assay preparation* and the *Standard preparation,* respectively.

Estrone Injection

» Estrone Injection is a sterile solution of Estrone in a suitable oil. It contains not less than 90.0 percent and not more than 115.0 percent of the labeled amount of $C_{18}H_{22}O_2$.

Packaging and storage—Preserve in single-dose or in multiple-dose containers, preferably of Type I glass.

Identification—Dissolve the residue obtained in the *Assay* in sufficient alcohol to obtain a solution containing 500 µg of estrone in each mL. Transfer to an acetylation flask, and evaporate to dryness. Add 10 mg of hydroxylamine hydrochloride, 0.20 mL of glacial acetic acid, and 5 mL of alcohol, and reflux for 5 hours. Dilute with 5 mL of water, filter, and recrystallize the precipitate from hot alcohol: the estrone oxime so obtained melts between 236° and 242°, the procedure for *Class I* being used (see *Melting Range or Temperature* ⟨741⟩).

Other requirements—It meets the requirements under *Injections* ⟨1⟩.

Assay—[NOTE—Use only water as a lubricant for the separators used in this assay, and complete the assay without interruption other than at the stage of obtaining the dry residue from the benzene extract.] Transfer a volume of Injection, equivalent to about 10 mg of estrone, to a suitable separator containing 25 mL, or not less than twice the volume of the Injection taken, of solvent hexane. Add 10 mL of sodium hydroxide solution (1 in 10), shake vigorously for 2 minutes, and allow the layers to separate completely. Transfer the aqueous layer to a second 125-mL separator, and repeat the extraction of the solvent hexane with two additional, successive 10-mL portions of the sodium hydroxide solution, adding each extract to the second separator. Complete the alkaline extraction as quickly as possible, since long standing in strongly alkaline solution may cause decomposition of the estrone. Wash the combined alkaline extracts with 25 mL of solvent hexane. Using dilute sulfuric acid (1 in 2), acidify the combined alkaline extracts until acid to litmus. Cool thoroughly, add 25 mL of benzene, shake carefully for 1 minute, and allow the layers to separate. Transfer the acid layer to another 125-mL separator, and extract with a second 25-mL portion of benzene. Discard the acid layer. Extract the benzene layers with two 5-mL portions of sodium carbonate TS and two 5-mL portions of water. Discard the aqueous layers. Transfer the benzene solutions to a beaker with the aid of benzene, and evaporate on a steam bath with the aid of a current of air to dryness.

Dissolve the residue from the benzene extract in a small quantity of chloroform, warming, if necessary, and completely transfer the solution, with the aid of a few mL of chloroform, to a 20- × 150-mm test tube. Carefully evaporate the chloroform on a steam bath with the aid of a current of air. Add 100 mg of trimethylacethydrazide ammonium chloride and 500 µL of glacial acetic acid to the test tube, insert the stopper loosely, and heat in a boiling water bath for 5 minutes. Cool the reaction mixture in an ice bath, dissolve in a small volume of cold water, and completely transfer, with the aid of a small volume of water, to a 125-mL separator containing 25 mL of cold water. Neutralize the solution to litmus with 1 N sodium hydroxide (approximately 6 mL), and wash at once with three 15-mL portions of chloroform. Combine the chloroform washings in another separator, and wash them with 5 mL of water. Discard the chloroform, and add the wash water to the first separator. Add 2 mL of dilute sulfuric acid (1 in 2), and allow to remain at room temperature for 2 hours. Add 15 mL of chloroform, shake vigorously for 1 minute, and allow the layers to separate. Transfer the chloroform layer to another separator, and repeat the extraction of the water layer with three additional, successive 15-mL portions of chloroform. Wash the combined chloroform extracts with 5 mL of water, filter through chloroform-washed cotton into a beaker, evaporate to a small volume, and transfer completely, with the aid of several small portions of chloroform, to a tared 25-mL beaker. Evaporate on a steam bath with the aid of a current of air to dryness, and dry the residue of estrone in a vacuum desiccator to constant weight: the weight of the residue, corrected for the residue of a reagent blank similarly prepared, indicates the amount of $C_{18}H_{22}O_2$ in the volume of Injection taken.

Estrone Injectable Suspension

» Estrone Injectable Suspension is a sterile suspension of Estrone in Water for Injection. It contains not less than 90.0 percent and not more than 115.0 percent of the labeled amount of $C_{18}H_{22}O_2$.

Packaging and storage—Preserve in single-dose or in multiple-dose containers, preferably of Type I glass.

USP Reference standards ⟨11⟩—*USP Endotoxin RS. USP Estrone RS. USP Progesterone RS.*

Identification—Transfer a volume of Injectable Suspension, equivalent to about 5 mg of estrone, to a glass-stoppered centrifuge tube, and add 2.5 mL of a mixture of ether and benzene (1 : 1). Shake for 2 minutes, and allow insoluble matter to settle, centrifuging, if necessary, to obtain a clear supernatant. Apply 5 µL each of this supernatant and a 1 in 500 solution of USP Estrone RS in a mixture of ether and benzene (1 : 1) to a suitable thin-layer chromatographic plate (see *Chromatography* ⟨621⟩), coated with a 0.25-mm layer of chromatographic silica gel. Allow the spots to dry, and develop the chromatogram in a solvent system consisting of a mixture of benzene and acetone (4 : 1) until the solvent front has moved about three-fourths of the length of the plate. Remove the plate from the developing chamber, mark the solvent front, and allow the solvent to evaporate. Spray the plate with a mixture of dehydrated alcohol and sulfuric acid (3 : 1), and heat in an oven at 105° for 10 minutes: the R_F value and appearance (pale orange to amber by direct observation in daylight, and fluorescing pale yellow-green under long-wavelength UV light) of the principal spot obtained from the test solution correspond to those obtained from the Standard solution.

Bacterial endotoxins ⟨85⟩—It contains not more than 88.0 USP Endotoxin Units per mg of estrone.

Uniformity of dosage units ⟨905⟩: meets the requirements.

Other requirements—It meets the requirements under *Injections* ⟨1⟩.

Assay—

Mobile phase, Standard preparation, and *Chromatographic system*—Prepare as directed in the *Assay* under *Estrone.*

Assay preparation—Transfer an accurately measured volume of the well-mixed Injectable Suspension, equivalent to about 10 mg of estrone to a 50-mL volumetric flask. Add 30 mL of methanol and swirl for 5 minutes. Dilute with methanol to volume, and mix. Transfer 5.0 mL of this solution to a 25-mL volumetric flask, dilute with *Mobile phase* to volume, and mix.

Procedure—Proceed as directed for *Procedure* in the *Assay* under *Estrone.* Calculate the quantity, in mg, of $C_{18}H_{22}O_2$ in each mL of Injectable Suspension taken by the formula:

$$0.25(C / V)(r_U / r_S),$$

in which V is the volume, in mL, of the Injectable Suspension taken, and the other terms are as defined therein.